



Morphological and molecular identification of an unknown fungal isolate from Al-Dujail District: A new record of *Tulostoma winterhoffii* in Iraq

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ABSTRACT The *Tulostoma* genus, known as stalkballs or stalked puffballs, belongs to the Agaricaceae family. This study was designed to identify an unknown fungal species collected from the Al-Dujail district in Iraq based on morphological examination and molecular analysis of the internal transcribed spacer (ITS) region. Between April and July 2019, samples were collected from garden soil in the Al-Dujail district, Salah Al-Din Governorate, Iraq. Morphological characteristics were documented using light microscopy. Genomic DNA was extracted and purified, and the ITS region was amplified using conventional PCR with specific primers. The amplified products were sequenced, and phylogenetic analysis was conducted using MEGA11 software. Morphological analysis revealed smooth, yellow to brown, nearly circular basidiospores. The ITS region amplification yielded a 588 bp fragment. Basic Local Alignment Search Tool (BLAST) analysis showed 91% similarity between the sample (S1-ITS-Iraq) and *Tulostoma winterhoffii* (accession number KU518975.1). The isolate was assigned in GenBank under accession number PV249065, with phylogenetic analysis positioning S1-ITS-Iraq in a cluster with the *Tulostoma* species, with a bootstrap value of 97%, indicating a close relationship. The fungal sample from Iraq was identified as a new record within the genus *Tulostoma*, marking the first report of *T. winterhoffii* in the region.

KEYWORDS Fungal DNA; Gene bank; ITS1; PCR; Phylogenetic tree; *Tulostoma winterhoffii*

1. Introduction

Fungi play a major role in ecosystems because of their biodegradation, plant symbiosis, medical and industrial applications. The genus *Tulostoma* belongs to the family Agaricaceae and is commonly known as stalkballs or stalked puffballs (Tomer et al. 2021; de Lima et al. 2023). Numerous studies have focused on *Tulostoma* species in arid and semi-arid regions, information about *Tulostoma winterhoffii* remains limited, particularly concerning its presence in humid environments and its effects on soils and ecosystems. This fungus was first defined in Germany in 1913 (Jeppson et al. 2017). No documented studies have been found on *Tulostoma* species in Iraq whereas 57 species had been reported in Asia (Paloi et al. 2023).

In mycology, there are two main methods required for identifying fungi to species level: morphological characteristics and molecular methods. Specially, sequence-based methods, by comparing the sequences of one or more genes in databases such as NCBI (National Center for Biotechnology Information), EMBL (European Molecular Biology Laboratory), DDBJ (DNA Data Bank

of Japan), or INSD (International Sequence Database). This strategy enables scientists to classify fungi to species level (Bich et al. 2021; Nuraini et al. 2023; Tarad et al. 2023).

This fungus has many contributions to soil fertility improvement through organic matter decomposition, or production of biological compounds (Bautista-González et al. 2022). So, studying this fungus increases knowledge of fungal diversity in Iraq. Therefore, this study was designed to identify an unknown fungal species collected from the Al-Dujail district in Iraq based on morphological examination and molecular analysis of the internal transcribed spacer (ITS) region.

2. Materials and Methods

2.1. Specimens collection

The fungal sample (fruiting body) was collected from the Al-Dujail district in Salah Al-Din Governorate in a period from April to July 2019; it grows on soil in the gardens during winter after the rains. The sample was transported

immediately to the laboratory for morphological examination according to Jeppson et al. (2017) and Finy et al. (2023).

2.2. Molecular diagnosis

Fungal DNA was extracted from the fruiting body using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. The total DNA was quantified using a Quantus™ Fluorometer (Promega) and stored at -20°C for further use. Fungal DNA was amplified by conventional PCR assay using primer set (ITS1): 5'-TCCGTAGGTGAACCTGCGG-3', ITS4: 5'-TCCTCCGCTTATTGATATGC-3'. The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide (Rusevska et al. 2019). The PCR amplicons were sent to Acrogen Corporation (Seoul, South Korea) for Sanger sequencing using an ABI 3730XL automated DNA sequencer. The resulting sequences were analyzed using the BLAST available at the NCBI website (<http://www.ncbi.nlm.nih.gov>) to define sequence similarity. A phylogenetic tree was created using MEGA11 software (Tamura et al. 2021) based on maximum likelihood method, and the Jukes-Cantor model with 1000 bootstrap replicates.

3. Results and Discussion

3.1. Results

The fungal fruiting body was characterized by a pear-colored body with a light brown color and thick roots that were inserted in the soil (Figure 1a). The fruiting body was cut into two parts and found to contain a yellow powder (Figure 1b). The fungal weight was between 300 to 400 g. It is brown with white on the top of the mushroom, resembling scattered snow. The basidiospores appeared smooth, yellow to brown and almost circular under a light microscope (40×) (Figure 1c).

The ITS region of the nuclear rRNA is a commonly used area for species and generic phylogenetic analysis. In this study, ITS1 and ITS4 primers were used to amplify DNA from an unknown fungal sample, resulting in a 588 bp fragment that was visualized under UV light using a transilluminator. The PCR product was separated on a 1% agarose gel by electrophoresis at 1 volt/cm^2 for 1 hour and stained with ethidium bromide. The ITS1 sequence was then matched against the GenBank database using the NCBI BLAST tool to identify related sequences. The results showed similarity, with S1-ITS-Iraq exhibiting 91% homology to the *T. winterhoffii* accession number in GenBank which was KU518975.1 (Table 1).

Phylogenetic analysis of the ITS1 region revealed the relationships among the fungal isolate S1-ITS-Iraq and related taxa. Figure 2 showed a phylogenetic tree that illustrates two primary clades. The first clade comprises *Geotrichum psychrophilum*, *Apiotrichum porosum*, *Dioszegia hungarica*, and *Helminthosporium velutinum*, exhibiting strong bootstrap support (98–99%). The second clade includes S1-ITS-Iraq and several *Tulostoma* species, with bootstrap values 97%. The cluster of three *T. winterhoffii* strains (KU518975.1, KU518976.1, and KU518977.1), showed bootstrap values 89%. When, these *T. winterhoffii* strains were grouped with *T. pseudopulchellum* and *T. submembranaceum*, showed the had low bootstrap values 48%.

3.2. Discussion

This study presents the new record of *Tulostoma winterhoffii* in Iraq. The fungal strain has been identified based on morphological and molecular analyses, the results showed a close phylogenetic relationship between the local isolate and *T. winterhoffii* sequences that have already been recorded in GenBank (KU518975.1). The morphological features observed, including the pear-shaped, light brown-colored fruiting body and round, yellow, powdery spores, are similar to descriptions from earlier studies on *Tulostoma* species (Finy et al. 2023; Jeppson et al. 2017). minor differences in spore size and color may be attributed to environmental factors, such as soil composition and temperature conditions in the Al-Dujail district.

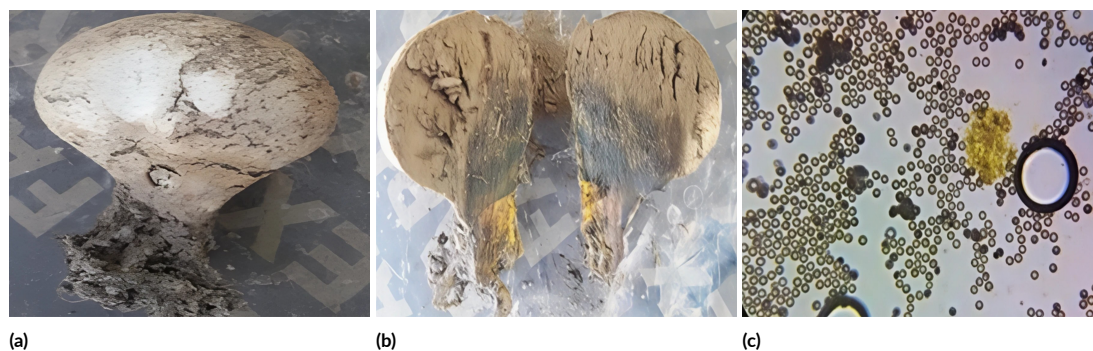


FIGURE 1 Macroscopic and microscopic characteristics of *Tulostoma winterhoffii* isolate. (a) Intact basidiocarp showing the pear-shaped morphology and light brown color. (b) Sectioned basidiocarp revealing the internal yellow, powdery spore mass. (c) Smooth basidiospores observed under a light microscope at 40× magnification.

TABLE 1 BLAST findings from the NCBI's GenBank database of the fungal isolates.

Isolates	Closest species in GenBank database	Identity	Accession number in GenBank	Isolate Accession No.
S1-ITS-Iraq	<i>Tulostoma winterhoffii</i>	91%	KU518975.1	PV249065

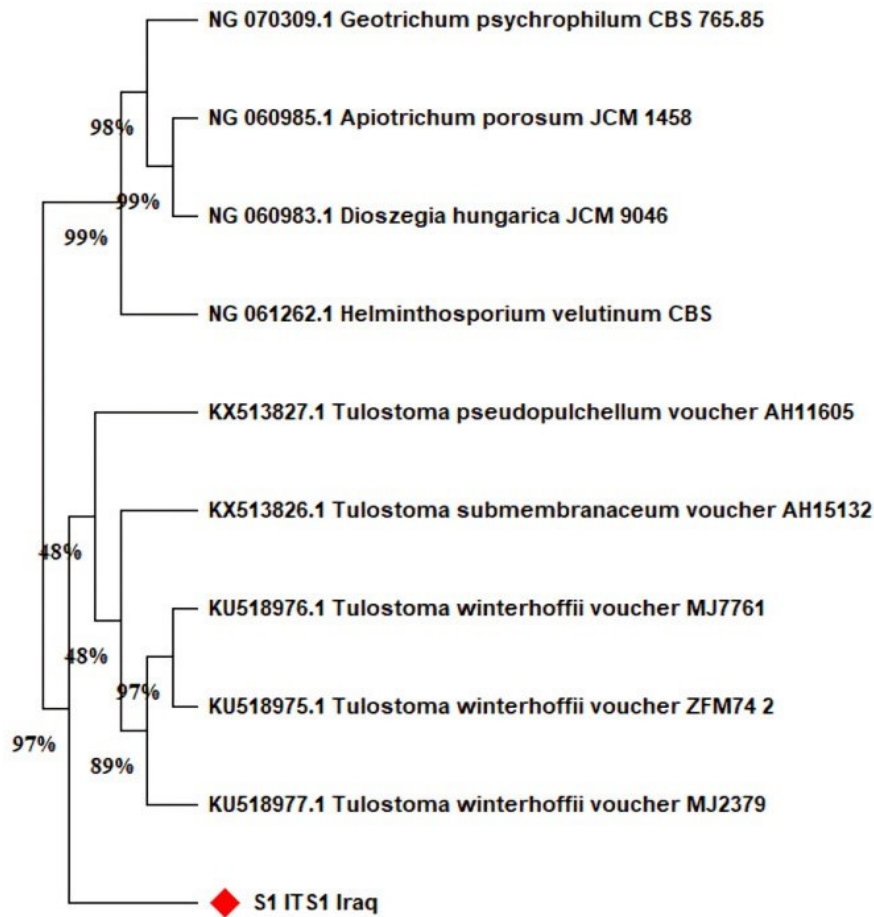


FIGURE 2 Phylogenetic analysis of ITS1 for unknown isolate in comparison with references sequences in GenBank database NCBI.

A study of the ITS region at molecular level confirmed the morphological findings, with the extended ITS1 sequence was 91% similarity to that *T. winterhoffii* (KU518975.1). Even though this similarity is significant, it still lower than the usual level ($\geq 97\%$) needed for precise species-level identification in fungal classification. This highlights the potential need for additional genetic markers or whole genome sequencing to confirm the isolate's species identity.

As mentioned previously, there is no study on the dissemination and growth of *T. winterhoffii* in Iraq; therefore, a biodiversity study of this fungus in this region is required. The ITS region was used a lot for identifying fungi at the species level (Gencheva and Beev 2021; Paloi et al. 2022; Jarjees et al. 2023); however, more loci are needed, like RPB2 (RNA polymerase II) or LSU (Large Subunit rDNA), to elucidate the principles underlying fungal taxonomy (Alhawatemala et al. 2019).

4. Conclusions

The present study identifies a fungus sample from the Al-Dujail district in Salah Al-Din Governorate, Iraq, as a new record genus, *Tulostoma*. However, further genetic and ecological investigations are needed to understand its classification and distribution.

Authors' contributions

TKM designed the study, HNA wrote the manuscript, SWM carried out the laboratory work, and MFJ analyzed the data. All authors read and approved the final version of the manuscript.

Competing interests

All authors declare that they have no competing interests.

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